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Comparison of Common Fluorescent Labels for LC/MS Analysis of Released N-Linked Glycans

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Abstract

- Analysis of N-glycans released from biotherapeutics frequently relies on the addition of a label/fluorophore.

- Using N-glycans released from MabThera and Enbrel, we compare the mass spectrometry (MS) and fluorescence response of N-linked glycans labeled with different fluorophores: Agilent (formerly ProZyme) InstantPC, InstantAB, 2-AB, procainamide, and 2-AA.

- Of the labels tested, InstantPC resulted in the brightest fluorescence and MS signal followed by procainamide, while 2-AB and 2-AA resulted in the lowest signal.

- The hydrophilic interaction liquid chromatography (HILIC) retention times and elution order of N-glycans labeled with InstantPC, procainamide, InstantAB, 2-AB, and 2-AA are compared.

- The comparison between MS and fluorescence response shows good agreement for InstantPC labeled glycans from MabThera.

- The favorable properties of InstantPC for MS analysis allow for the detection of low abundance glycans.

- An additional benefit of InstantPC is the instant nature of the glycosylamine-reactive dye, which offers a faster workflow and time to results compared to traditional reductive amination when using other dyes.

Introduction

The glycosylation pattern of biotherapeutic proteins is a critical quality attribute that impacts immunogenicity, pharmacokinetics, and pharmacodynamics.[1] Carbohydrates and glycans do not contain a chromophore/fluorophore suitable for online detection with standard liquid chromatography techniques (UV/fluorescence), and their mass spectrometry (MS) response is generally poor due to their low ionization efficiencies. Consequently, glycans are commonly derivatized prior to their analysis to increase their detectability. Traditional and commonly used fluorescent dyes 2-AB, 2-AA,[2] and procainamide[3] involve reductive amination through Schiff base formation. This approach often requires multiple steps including drying released glycans prior to labeling and a heated labeling reaction, which can take hours if not days to complete.[4] The Agilent InstantDyes (formerly ProZyme) such as InstantPC[5] and InstantAB[6] react rapidly with glycosylamines released after PNGaseF digestion, resulting in much faster labeling and time to results (Figure 1).

To compare the fluorescence and MS response of these dyes, N-glycans from monoclonal IgG MabThera (rituximab) and Fc-fusion protein Enbrel (etanercept) were released using the Agilent GlykoPrep Rapid N-glycan Preparation system (formerly ProZyme) and labeled with InstantPC, procainamide, InstantAB, 2-AB, and 2-AA.

Materials and methods

Sample preparation

N-Glycan samples from MabThera (lot no. B6055B01) and Enbrel (lot no. 1063858) were prepared using Agilent GlykoPrep protocols (formerly ProZyme) using 50 µg of glycoprotein per preparation with individual dyes InstantPC, procainamide, InstantAB, 2-AB, and 2-AA. (Table 1). Excess dye was removed with Agilent GlykoPrep cleanup cartridges (formerly ProZyme), and eluted samples were adjusted to a final volume of 50 µL prior to HILIC-UHPLC analysis (1 µL injection) with appropriate elution buffer.

HILIC-UHPLC MS analysis

Glycans were separated by hydrophilic interaction liquid chromatography (HILIC). UHPLC-HILIC separation was performed on an Amide column (2.1 x 150 mm, 1.7 µm) using a 25 to 38% 50 mM ammonium formate pH 4.4, between 2.5 and 50 minutes at a flow rate of 0.4 mL/min. The flow was coupled to a Q-TOF and analysis was performed in the positive mode using a capillary voltage of 2.8 kV, cone voltage 30 V, source temperature 120 °C, desolvation temperature 350 °C, scan time 0.8 second, and *m/z* range 400 to 2000 Da.
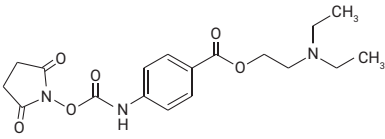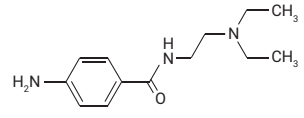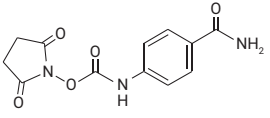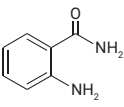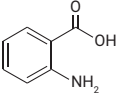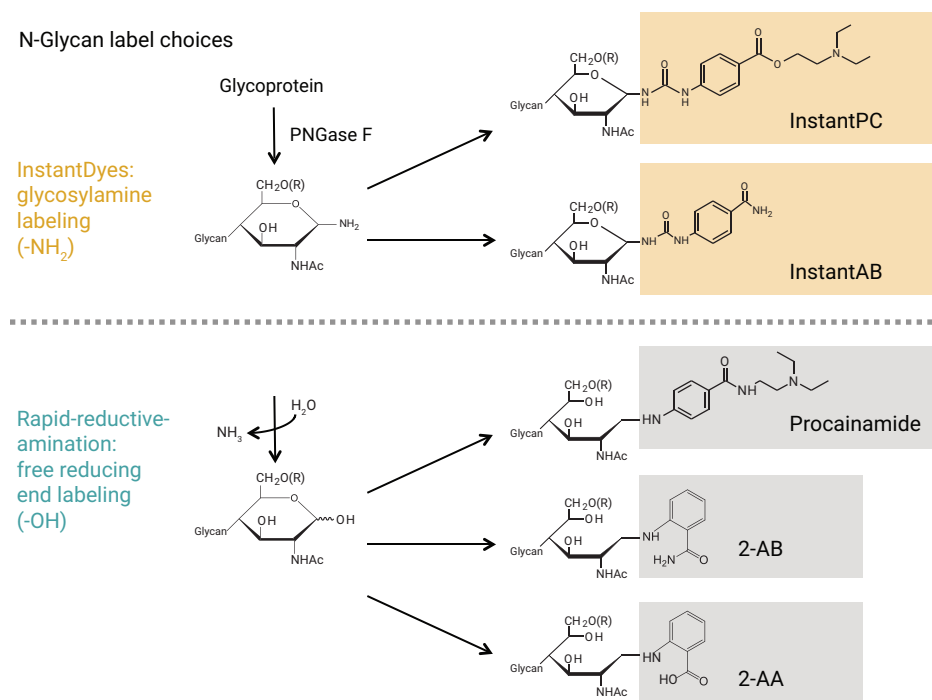

Figure 1. Comparison of Instant dye labeling and traditional reductive amination. InstantDyes react with glycosylamines released by PNGase F, while reductive amination proceeds the free reducing end hydroxyl (-OH).

Online fluorescence detection

Labeled glycans were monitored by fluorescence detection with appropriate excitation (Ex) and emission (Em) wavelengths (Table 1) using an UHPLC fluorescence detector (FLD).

Table 1. Dye Structures with fluorescence excitation (Ex) and emission (Em) wavelengths

| Labeling Fluorophore	λ Ex/Em (nm)	Dry Down Step	Labeling Time
InstantPC (5)	285/345	No	1 minute
Procainamide (3)	308/359	Yes	>1 hour
InstantAB (6)	278/344	No	1 minute
2-AB (2)	360/428	Yes	>1 hour
2-AA (2)	352/435	Yes	>1 hour

Results and discussion

UHPLC fluorescence performance

HILIC-UHPLC analysis of labeled N-glycans results in separation of the major glycans for both MabThera (Figure 2A) and Enbrel (Figure 2B).

The fluorescence intensities of equivalent quantities of glycans loaded on column varies by the dye used for labeling (Figure 2A inset).

Using the same UHPLC method, 2-AB-labeled glycans elute first, followed by 2-AA, InstantAB, InstantPC, and procainamide (Figure 2).
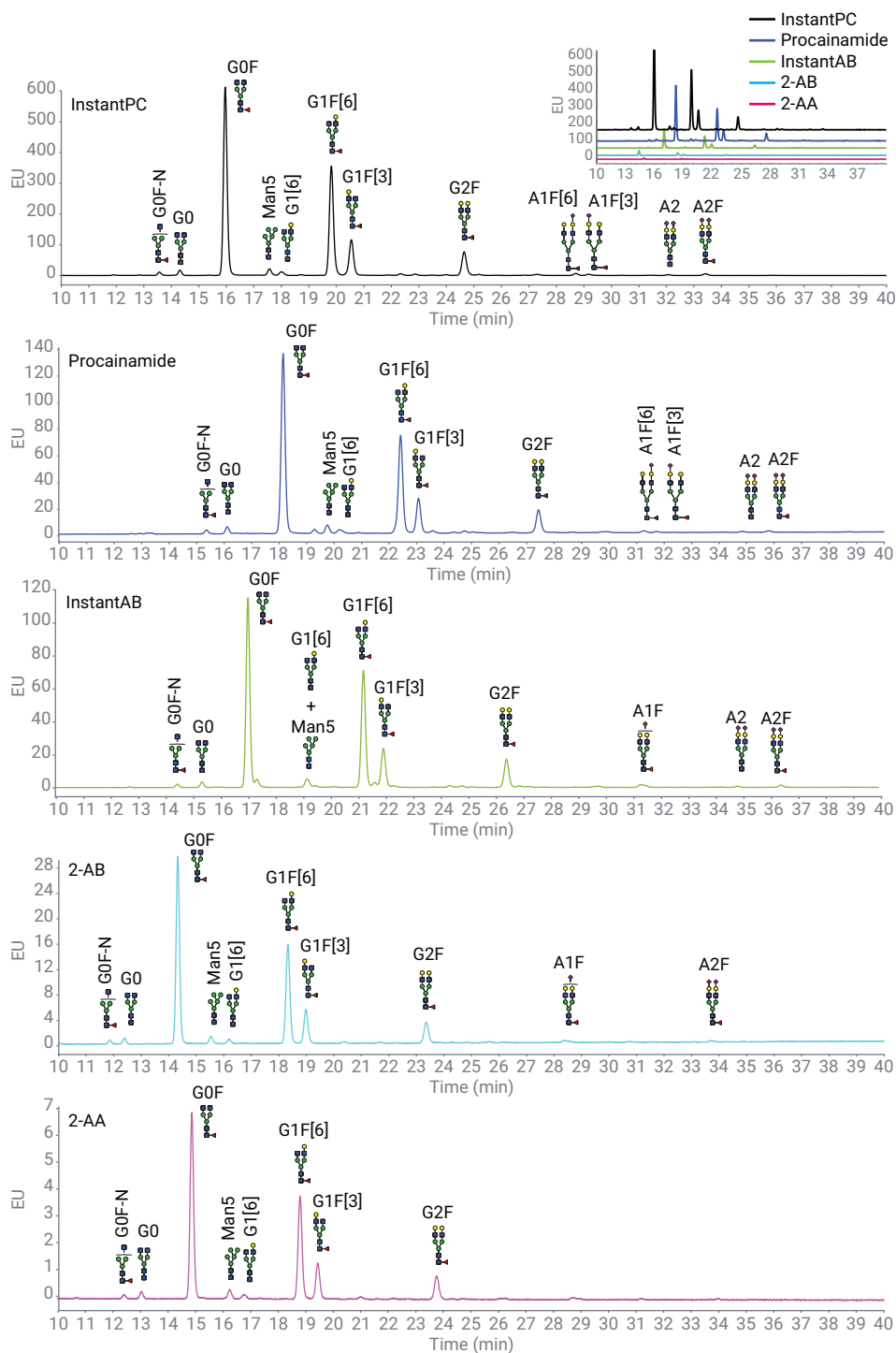
Figure 2A. UHPLC fluorescence profile of MabThera N-glycans labeled with Agilent InstantPC, procainamide, Agilent InstantAB, Agilent 2-AB, and 2-AA. Equal amounts of glycoprotein were prepared with Agilent GlykoPrep and labeled with each respective individual dye.

Resolution differences are observed with the different dye-labeled glycans. For example, only InstantPC- and procainamide-labeled samples result in the separation of both regions where Man5/G1[6]/G1[3] and A1F[6]/A1F[3] elute. Procainamide has the drawback of not resolving G2F/G1FS1, as observed with Enbrel N-glycans (Figure 2B) in addition to the observation of minor artifact peaks resulting from the developmental procainamide protocol.[3]
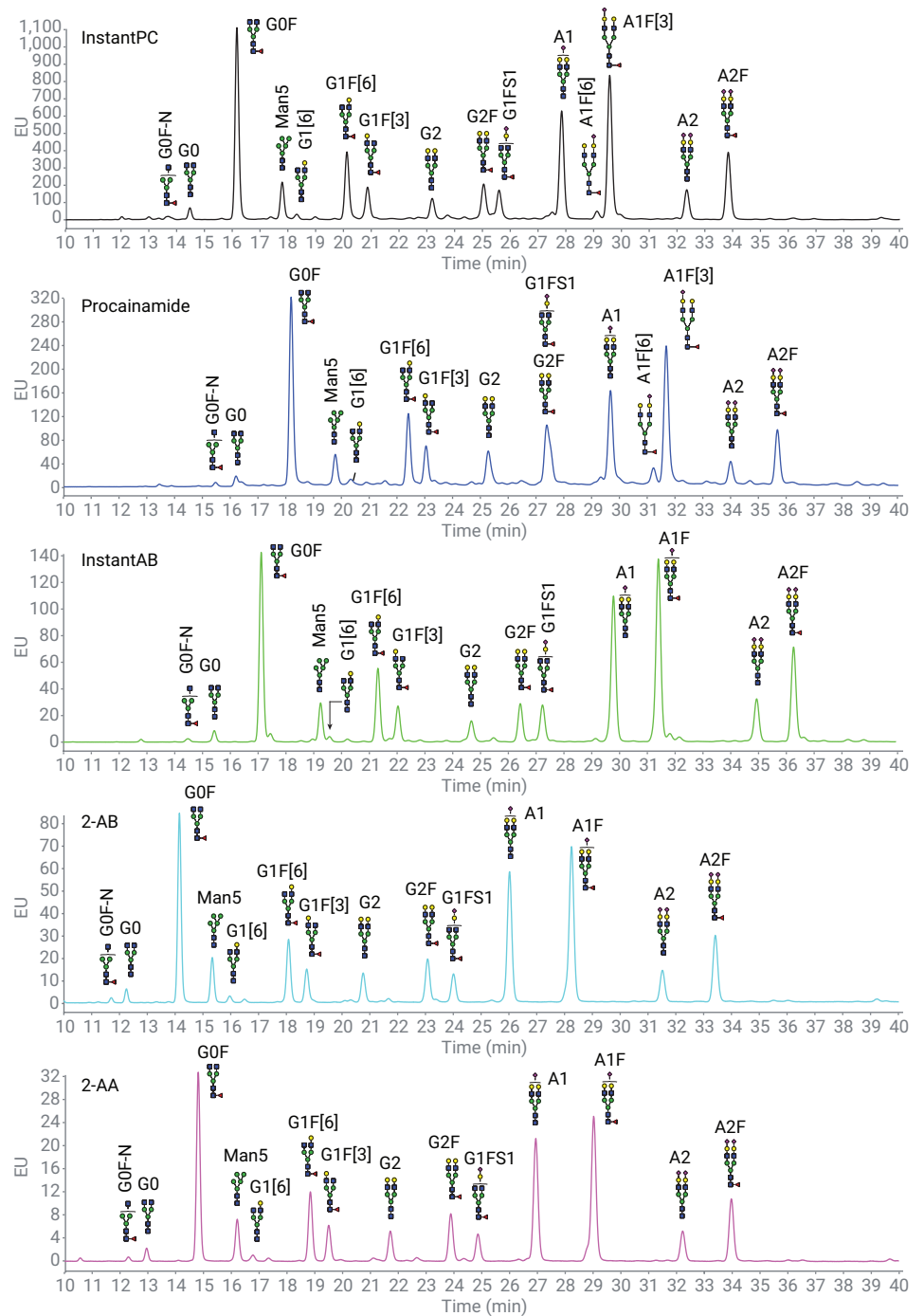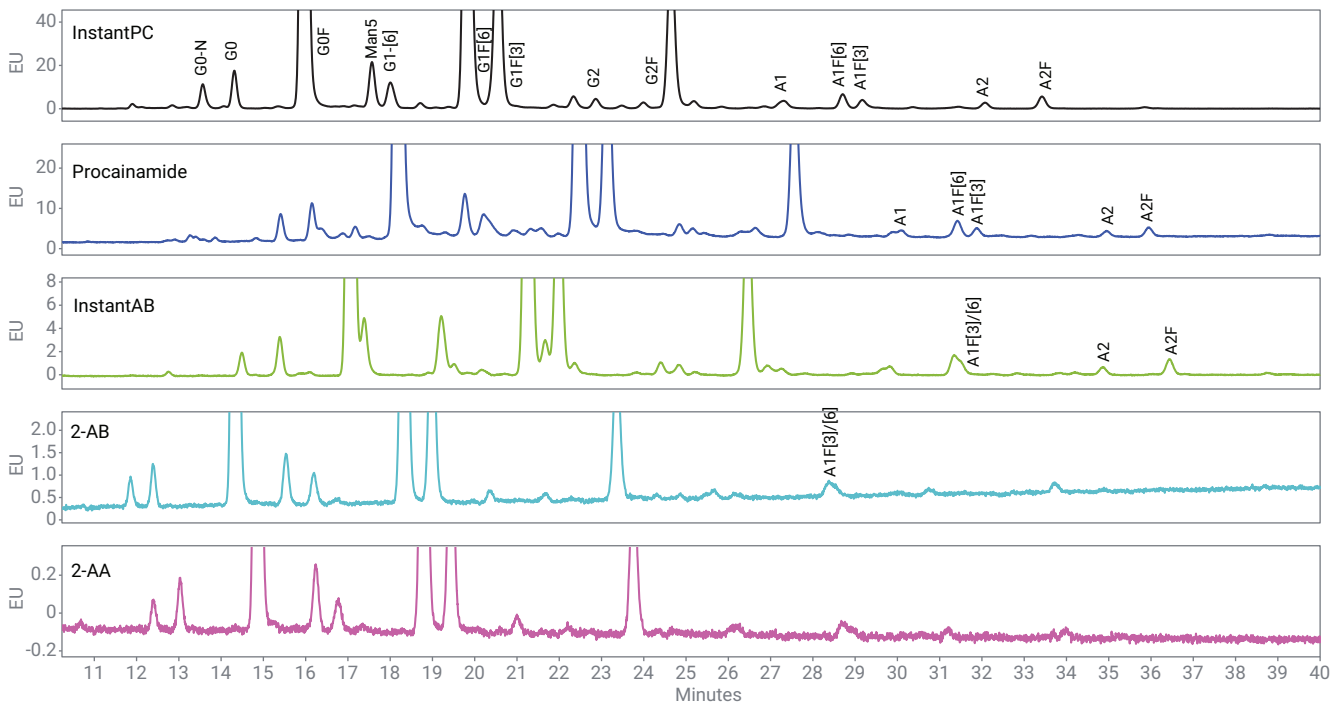

Figure 2B. UHPLC fluorescence profile of Enbrel N-glycans labeled with Agilent InstantPC, procainamide, Agilent InstantAB, Agilent 2-AB, and 2-AA. Equal amounts of glycoprotein were prepared with Agilent GlykoPrep and labeled with each respective individual dye.

The strong fluorescence signal of InstantPC compared to traditional labeling dyes 2-AB and 2-AA allows for the detection of low abundance N-glycans, as observed when comparing MabThera N-glycans labeled with different dyes (Figure 3).

The fluorescence peak area of InstantPC-labeled G0F is nearly two times the next most intense signal for procainamide (Figure 4), which requires reductive amination and a much longer sample preparation time.
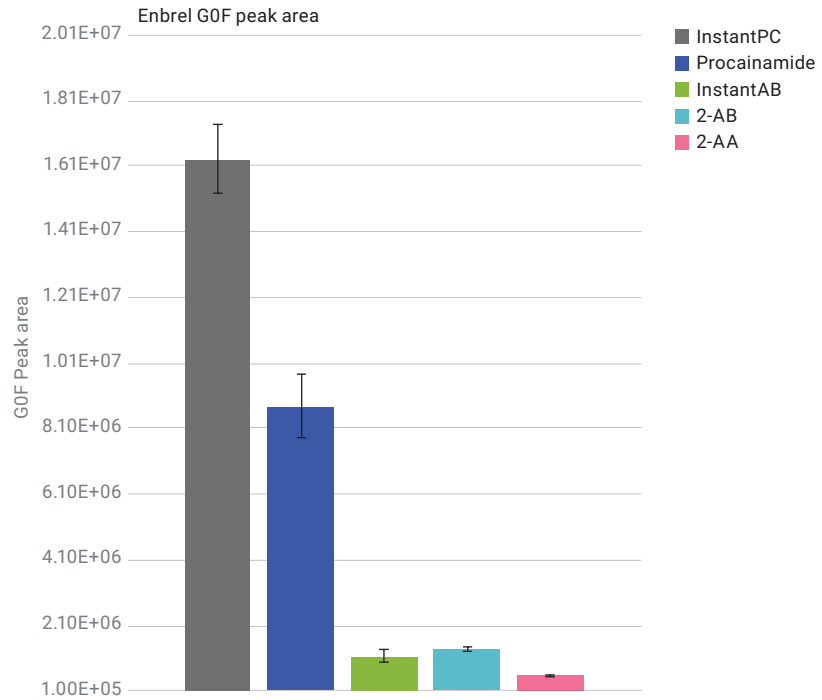
Figure 4. Comparison of fluorescence response. Glycans from equivalent amounts of glycoprotein were prepared with Agilent GlykoPrep, labeled with Agilent InstantPC, procainamide, Agilent InstantAB, Agilent 2-AB, and 2-AA. Samples were analyzed by HILIC-UHPLC.
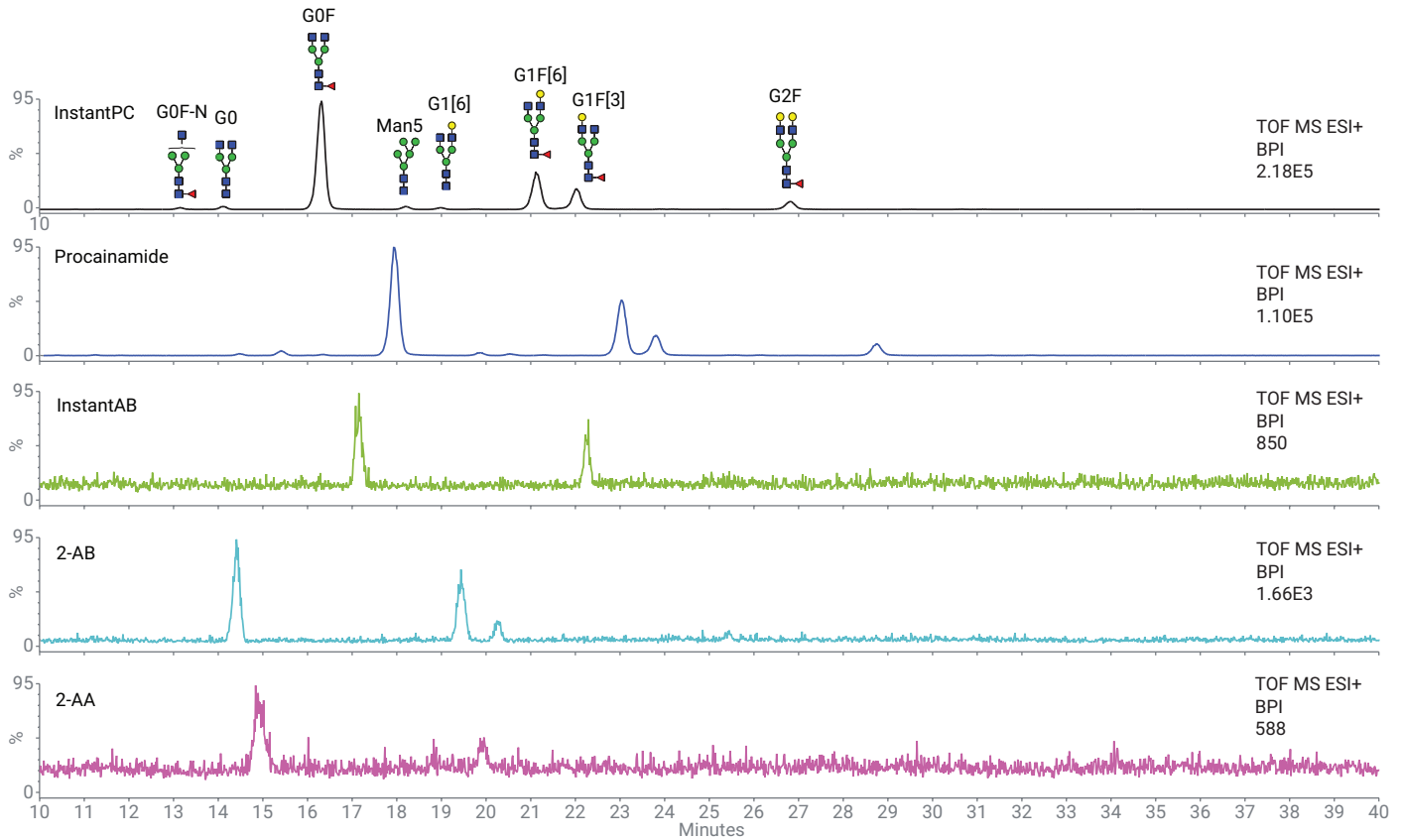

Figure 3. Zoomed in fluorescence profile of MabThera N-glycans labeled with Agilent InstantPC, procainamide, Agilent InstantAB, Agilent 2-AB, and 2-AA. InstantPC and procainamide-labeled N-glycans allow for the detection and resolution of low abundance glycans A1F[6] and A1F[3], while InstantAB, 2-AB, and 2-AA are not baseline resolved, and do not resolve A1F[3]/[6].

MS Performance

Figure 5 shows the base peak chromatogram (BPC) obtained with MabThera N-glycans-labeled with InstantPC, procainamide, InstantAB, 2-AA, and 2-AB. InstantPC yields the highest MS response of all dyes examined.

Detection of A1F[6]/[3] by MS was only observed with InstantPC and procainamide (Figure 6A extracted ion chromatogram, 6B mass spectrum for A1F $[M+2H]^{2+}$).

Figure 5. Comparison of base peak chromatograms (BPC). Glycans from equivalent amounts of MabThera were prepared with Agilent GlykoPrep, labeled with Agilent InstantPC, procainamide, Agilent InstantAB, Agilent 2-AB, and 2-AA. Samples were analyzed by HILIC-UHPLC-MS.
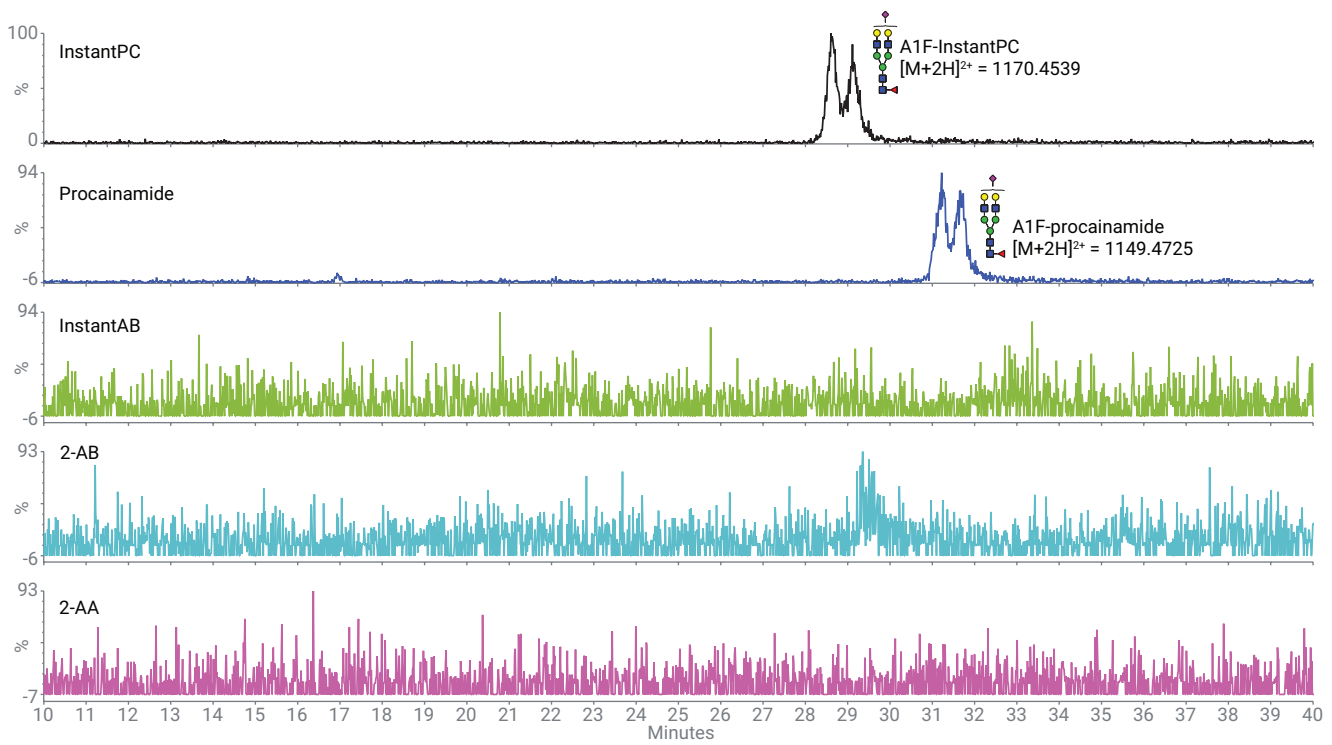
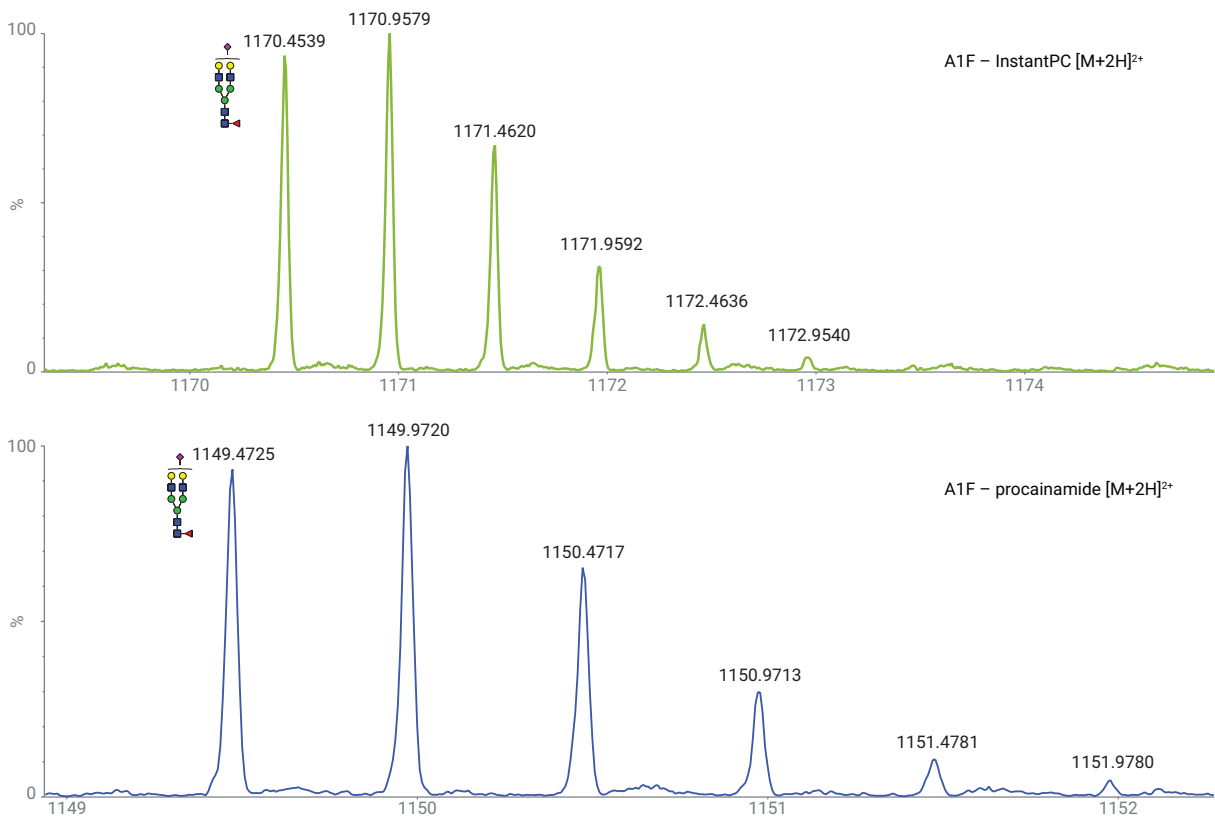**Figure 6A.** Extracted ion chromatogram A1F $[M+2H]^{2+}$.

Figure 6B. Mass spectrum of Agilent InstantPC- and procainamide-labeled A1F glycan from MabThera.

An additional benefit of InstantPC is the close correlation between fluorescence and MS peak areas when calculating relative percent areas (Figure 7).

Conclusion

- Agilent InstantPC results in the highest MS and fluorescence signal of all dyes examined.

- Glycan labeling dyes have differences in HILIC-UHPLC retention and selectivity, however retention order remains relative with InstantPC, procainamide, Agilent InstantAB, Agilent 2-AB, and 2-AA.

- InstantPC labeling of N-glycans allows for the detection and resolution of sialylated and low-abundance glycans by fluorescence and MS.

- Detection of low-abundance glycan A1F[6]/[3] by MS was only observed with InstantPC and procainamide (Figure 6A extracted ion chromatogram, 6B mass spectrum for A1F$[M+2H]^{2+}$).

- InstantPC-labeled N-glycans show close agreement between MS and fluorescence response, facilitating relative quantification.

- InstantPC uses instant chemistry, allowing for much faster sample prep workflow compared to traditional reductive amination methods.

FLD versus MS relative % area
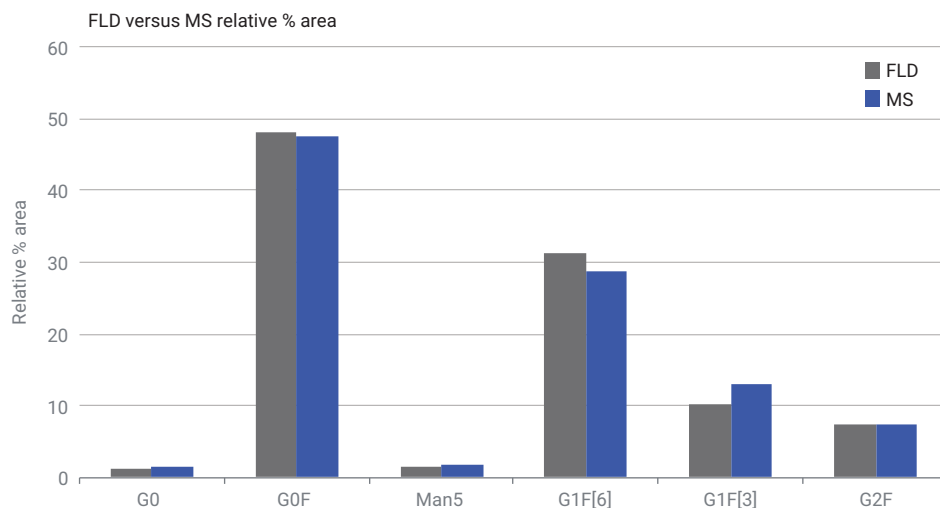
Figure 7. Comparison of fluorescence versus MS peak areas for Mab-Thera N-glycans labeled with Agilent InstantPC. MS peak areas represented are sum totals of charge states $[M+2H]^{2+}$, $[M+NH_4+H]^{2+}$, and $[M+Na+H]^{2+}$.

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